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The Antiviral Effects of Embelin on HSV-1 in Cultured Vero Cells

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Abstract

Herpes simplex virus type 1 (HSV-1) is a linear double stranded DNA virus that is responsible for one of the most common sexually transmitted diseases within the United States. However, treatment drugs for HSV-1 could be costly and may have side effects. In recent years the number of cases of drug resistant HSV-1 has increased, resulting in interest for novel treatments. This study aimed to investigate the antiviral effects of the reagent Embelin on HSV-1 infection in cultured Vero Cells. Embelin is a benzoquinone isolated from the plant *Embelia ribes*. Cell viability and cell proliferation assays were conducted to determine the maximum noncytotoxic concentration of Embelin. Additional assays were conducted to measure cell proliferation and antiviral effect. The results of this study suggest that Embelin indeed inhibits HSV-1 with best viral inhibition between 50 μ M and 54 μ M. Also, Embelin seems to be most effective at inhibiting the virus at the binding stage of the viral cycle.

MONTCLAIR STATE UNIVERSITY

THE ANTIVIRAL EFFECTS OF EMBELIN ON HSV-1 IN CULTURED VERO CELLS

by

Tony Elias

A Master's Thesis Submitted to the Faculty of

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In Partial Fulfillment of the Requirements

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Montclair, NJ

2020

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Review of literature

Classification and life cycle

HSV-1 is a member of the family *Herpesviridae*, and subfamily *Alphaherpesvirinae*. *Alphaherpesvirinae* is special due to the rapid production and the quick tearing down of the host cell (Mettenleiter et al., 2008). HSV-1 is double stranded DNA (dsDNA) enveloped virus that causes a wide range of infections to humans from mild uncomplicated mucocutaneous infections to life-threatening ones (Whitley and Roizman, 2001). HSV-1 has a 125-Kb linear genome that encodes for no less than 74 genes within the genome, however overlapping transcripts, allows for as many as 84 different proteins (McGeoch et al., 2006; Rajčani et al., 2004). Furthermore, the genome of HSV-1 contains two regions, a long region called long unique region (UL) and a short one called the short unique region (US). From the 74 known genes found in HSV, 54 of those genes are found in UL where the US contains 12 (McGeoch et al., 1988). The size of the virus is relatively large compared to other viruses. The dsDNA is enclosed within an icosahedral protein which is surrounded by a lipid bilayer (Mettenleiter et al., 2006). HSV-1 is one of the most successful human pathogens in terms of its worldwide spread and persistence in the host. HSV-1 has become a global health issue with 67% (estimated 3.4 billion) of the world population between the ages of 0 and 49 infected (Looker et al., 2015). Studies have also shown an observed increase in neonatal spread occurrence between 2009 and 2015 in the Medicaid population in the United States (Szpara et al., 2014; Mahant et al., 2019).

Entry of the virus involves multiple surface glycoproteins, glycoprotein B (gB), glycoprotein C (gC), glycoprotein D (gD), glycoprotein H (gH), and glycoprotein L (gL), interacting with surface receptors on the host cell. Specifically, gC and/or gB binds to surface particles followed by gD binding to entry receptors (Clarke, 2015; Subramanian and Geraghty, 2007; Stevens, 1975). However, gC is not essential for the entry of the virus, but lacking it decreases entry and binding of the virus to the host cell (Shukla and Spear, 2004). Both gB and gH show fusion characteristics, while gD does not (Campadelli-Fiume et al., 2007; Heldwein et al., 2006). A number of cell receptors were found that interact with gD. Some of these known receptors include heparan sulfate, nectin-1, nectin -2, and herpes virus entry mediator (Spear, 2004). After one of these cellular receptors binds to gD, causing a conformation change, a glycoprotein complex that includes gB, gD, gH and gL forms (Tiwari and Shukla, 2010). This causes the mixing of the membranes followed by entry and binding of the virus to the minus end of the microtubule dynein (Sodeik et al., 1997). The virus then travels to the nucleus where it injects the viral DNA (Newcomb et al., 2007). This process occurs using a capsid portal made from 12 copies of portal protein (UL6) (Newcomb et al., 2007; Cardone et al., 2007). Transcription of the HSV-1 DNA is done using the enzyme RNA polymerase II from the host cell (McGeoch, 2006). The first genes that are transcribed, immediate early genes, express proteins that regulate the synthesis of early, late and latency stage proteins (Adang et al., 2006). These genes are transcribed immediately following the infection (Murphy et al., 2008). The late genes codes for the capsid and its receptors. After assembly, the virus then buds out of the cell membrane forming a membrane around the capsid (Granzow et al., 2008).

The scope of the problem

HSV-1 is the viral causative agent of genital and oral herpes. These are contagious and long-lasting infections that cause painful blisters or ulcers at the site of infection (McQuillan, 2018). According to the World Health Organization, approximately 3.7 billion people or 67% of the world's population under the age of 50 are infected with HSV-1, and more than 50% of adults in the United States are seropositive (Hoshino et al., 2009). In younger females, the rate of genital HSV-1 infection has become more common than the rate of HSV-2 genital infection (Bernstein et al., 2013). Herpes infection most commonly occurs in the Americas, Europe and Western Pacific according to the World Health Organization. Since the virus can remain latent, it can be reactivated at any time well into adulthood.

There are many cases of which a possible complication may occur. Although these complications might be rare, the over prevalence of the virus makes it otherwise. A good example is Herpes viral encephalitis, or Herpes simplex encephalitis (HSE), a form of encephalitis that is due to herpes simplex infection. Although both HSV-1 and type 2 can cause this encephalitis around 90% of cases of herpes encephalitis are caused by HSV-1 (Bradshaw and Venkatesan, 2016). HSE has been associated with 30% mortality as well as severe neurological defects in treated patients with 70% mortality in untreated patients (Zatoński et al., 2006). Symptoms of HSE develop over several days (Gnann and Whitley, 2017). Early symptoms include headaches, fevers, and seizures. More advanced symptoms include aphasia, anosmia as well as memory loss (McGrath et al., 1997).

Another complication is recurrent ocular infection. This ocular infection, also known as Herpetic simplex keratitis, is the leading cause of infectious blindness in developed countries, with around 40,000 new cases of severe visual impairment arising every year (Farooq and Shukla 2012). In addition, neonatal herpes is becoming more prevalent with around 1,500 cases of neonatal herpes being reported yearly in the United States, but most occur in Africa (George et al., 2014; Looker et al., 2017). Neonatal herpes is the cause of transmission of HSV-1 from the mother exhibiting the genital infection to a child during birth (Linakis and Reynolds, 2020). Finally, a study done in 1980 has shown some link between HSV-1 and Alzheimer's disease (Middleton, 1980). HSV-1 seems to harm the nervous system and interact with components and receptors of lipoproteins, which may lead to the development of Alzheimer's disease (Mosmann et al., 2019). However, according to a study published in 1997, there seems to be no neurological damage or increase the risk of Alzheimer's if the patient lacks the epsilon4 allele of the gene APOE (Itzhaki et al., 1997). In addition, another study published in 2008 showed a statistical difference between individuals with antibodies against HSV, indicating recent reactivation, and those without these antibodies in the incidence of Alzheimer's disease (Letenneur et al., 2008). Yet, it is worth noting the patients studied had no antibody at baseline making the result open to question. Another study done in the United Kingdom showed that treating HSV1-infected cells with antiviral drugs decreased the accumulation of β -amyloid and tau proteins that are linked to Alzheimer's disease (Wozniak et al., 2011). Finally, a more recent 2018 retrospective study from Taiwan conducted on 33,000 patients with HSV-1, showed that HSV-1 infection increased the risk of dementia 2.56 times for the untreated patients and no elevated risk of dementia for

the treated ones (Tzeng et al., 2018). This high prevalence and complications associated with the virus indicates the scope of the problem and the amount of attention it requires.

Herpes virus is transmitted by contact with an infected person who has reactivation of the virus (Dobson and Itzhaki, 1999). Also, asymptomatic reactivation may occur in which the virus causes atypical, hard to notice, or minute symptoms that are not identified as an active herpes infection or disease; therefore, acquiring the virus is possible even if no active HSV blisters or sores are present (Cohrs et al., 2008). The infection of HSV-1 can be active (lytic), or dormant (lysogenic) (Placek et al., 2010). The reactivation of HSV-1 can be due to either stress, immunosuppression, radiation or even heat (Halford et al., 1996). When the reactivation is triggered in some way, the virus will travel along the neuronal axon and re-infect the epithelial cells. This process of dormant and reactivation is the cause of the lifelong symptoms of HSV-1 over the patient's lifetime.

Treatment

There is no cure for Herpes simplex virus. The most common treatment of HSV-1 involves acyclovir which is a nucleoside analog, its derivatives and their respective prodrugs, valacyclovir and famciclovir (James et al., 2014; Tyring et al., 2000). These drugs function as DNA chain terminators by targeting DNA polymerase and therefore, they decrease viral propagation by the suppression of viral replication (Piret and Boivin, 2011).

The ability of the virus to exist in the latent stage reveals the inefficiency of these treatments (Agelidis and Shukla, 2015). In addition, the virus can easily gain resistance to

these types of drugs (Liang et al., 2018). Lastly, there is a noticeable increase in the global load of HSV-1 rather than a decrease, shedding light on the need for additional treatment strategies (Durukan et al., 2019). Therefore, there is an urgent need for new treatments against the virus.

In recent years, there has been a strong effort in developing an HSV vaccine (Royer et al., 2015). These efforts have focused mainly on recombinant viral proteins, attenuated viral candidates and hybrid recombinant viruses (Sandgren et al., 2016). Many vaccines have been developed, starting from the 1920s, yet none has been successful to this day (Alami Chentoufi et al., 2012). A successful vaccine should induce immune responses adequate to prevent infection, prevent colonization of the ganglia or at least reduce the frequency or severity of recurrences of viral episodes (Sandgren et al., 2016). Due to the high genetic similarity of both herpes simplex virus type 1 and 2 (Lasky and Dowbenko, 1984), a prophylactic or therapeutic vaccine that is effective against one type could also prove to be effective against the other.

There are many vaccines in clinical trials (Denes et al., 2020). However, there are multiple reasons and contributing factors for the failure to develop successful vaccines. One of those reasons is the fact that Herpesvirus is able to evade many features in the human immune response (Su and Zheng, 2016). The subunit vaccines which use small groups of viral antigens are promising. However, these are generally limited to producing humoral immunity (Ashley et al., 1985). Attenuated vaccines and replication-defective vaccines can produce both cellular and humoral immunity (Nguyen et al., 1992). However, they proved challenging to produce at scale offering limited immunization (Arvin et al., 2007).

Another very important contributing factor in the partial failure of recent vaccine trials, is the wide genetic variation in strains of HSV-1 (Szpara et al., 2014). An important target for vaccines is the viral glycoprotein B. This has been the target for many vaccination studies due to it being a prominent antigenic site. Its first 80 amino acids sequence acts as a binding site for MHC II proteins on antigen-presenting cells (Sievers et al., 2002). Yet, some studies have shown that these sites are highly variable sites, explaining the challenges present in the production of vaccines (Szpara, et al., 2014). Alternative treatments such as microbicide and specifically those of natural products seem promising. In recent years, research has shown that natural products can exhibit remarkable antiviral activity (Li et al., 2018; Asai and Nakashima., 2018).

Natural products

Natural products have been and continue to be a vital source of medical treatments. These natural products are very important because they allow for economic and environmentally friendly production minimizing the harmful toxins and by products (Ji et al., 2015). The vast majority of current medicines are either directly obtained natural products or derived (Atanasov et al., 2015). More importantly in this case, a large spectrum of antivirals has been isolated from fruiting bodies and filtrates of various biological plants and mushrooms (Pradeep et al., 2019). In addition, many biological plant based compounds have been demonstrated to hold activity against HSV-1 (Hassan et al., 2015). There have been many discoveries in recent years highlighted by plant based compounds such as curcumin, resveratrol, pomegranate rind extract, *Cornus canadensis* extract, and extracts from *Camellia sinensis*, epigallocatechin gallate

(EGCG), palmitate-epigallocatechin gallate (P-EGCG) and epigallocatechin gallate - stearate (EGCG-S) against HSV-1.

Curcumin, the main bioactive compound in turmeric, has shown antiviral activity against HSV-1 (Zandi, 2010; Flores et al., 2016). Resveratrol, the main bioactive compound in red wine (Annunziata et al., 2018). In several studies done in vivo, resveratrol significantly reduced lesions in a dose, start of treatment time, and number of applications dependent manner (Docherty et al., 2004; Docherty et al., 2005). It has been reported that 30 μ M of curcumin reduces the production of both HSV-1 and HSV-2, this occurs through interfering with the adsorption step of the viral cycle (Flores et al., 2016). Other compounds such as Punicalagin and ellagic acid, which are the active compounds in pomegranate rind extract, have presented antiviral activity against HSV-1.

Hydrolysable tannins have also shown some antiviral effects against HSV-1 (Houston et al., 2017). Hydrolysable tannins are the active chemical in *Cornus canadensis* extract. Studies demonstrated that at a concentration of 100 μ g/mL HSV-1 is inhibited in both the replication and absorption steps (Lavoie et al., 2017).

Crude extracts have been shown to obtain some antiviral activity encompassing anti- HSV-1 activity (Schnitzler et al., 2010). For example, various studies have shown that the resin collected by bees from plants (propolis) has shown promising inhibitory activities. It has shown over 50% inhibition in *vitro* studies and 80-85% inhibition in vivo (Huleihel and Isanu, 2002). It has been suggested that the presence of polyphenols, flavonoids and phenyl carboxylic acid are the reasons for the inhibition activity (Schnitzler et al., 2010).

EGCG is one of the four polyphenols present in green tea reported to have many biological activities including antioxidant and anticancer (Wolfram, 2007). EGCG has been shown to process many antiviral activities against a wide range of viruses (Vázquez-Calvo et al., 2017). From these, EGCG has shown to inhibit both HSV-1 and HSV-2 (Calland et al., 2012). However, EGCG is not stable. A modified EGCG has shown some very promising results against HSV-1. For example, digallate dimers of EGCG have been shown to inhibit HSV-1 under 100 μ M concentration (Isaacs et al., 2011). A more stable form of EGCG, EGCG-S, inhibits the HSV-1 (Paschalis, 2019). Finally, P-EGCG also inhibits the virus at concentrations of 50 μ M and higher (De Oliveira et al., 2013).

It is important to continue to search for novel antiviral compounds due to the ability of the virus to develop resistance to therapeutics (Robert et al., 2014). It is especially important because the virus infects different cell types in humans. Embelin a novel compound has shown some promising biological activities including anticancer, anti-inflammatory, and antibacterial (Kumar et al., 2017). Minimal work has been done on its antiviral effects and no studies have been done on HSV-1 (Pullaiah, 2002).

Embelin

Embelin (2,5-dihydroxy-1,4-benzoquinone) is a benzoquinone derivative that exhibits anxiolytic, anti-inflammatory activity, antioxidant, anticonvulsant, antidepressant, anthelmintic, antimicrobial, anticancer and many more biological activities (Kumar et al., 2017). Embelin is an orange solid derived from the plant *Embelia ribes* found throughout India. It is not water soluble; however, it can dissolve in organic solvents. Embelin is not evaluated as a hazardous substance (Radhakrishnan and

Gnanamani, 2014). Many animal experiments have shown no significant changes in behavioral or physical appearance upon Embelin treatment in small dosage (Prakash et al., 1994). Additionally, Embelin has been shown to have a high therapeutic index (as high as 200mg/Kg) in some studies (Gupta, 1991).

Embelin is known for its anticancer activity (Xu et al., 2005). It has been shown that it can act on caspase proteins to induce apoptosis (Joy et al., 2010). Also, it can improve the function of the liver and act as an antioxidant (Singh et al., 2009). Embelin has shown some analgesic effects as an anti-inflammatory (Chitra et al., 1994). Embelin is considered to be a good candidate for treating skin diseases as well as respiratory disorders, helminthiasis and gastrointestinal disorders as well as improving immune functions and blood circulation (Poojari, 2014; Kumar, et al., 2011).

Embelin has been studied as an antibacterial agent. In one study, it has been shown to have a significant reduction against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Shigella flexneri*, *Shigella sonnei* and *Pseudomonas aeruginosa* (Chitra, et al., 2003). A more recent study has shown that Embelin provides promising antibacterial activity against some Gram-positive bacteria and bacteriostatic activity against Gram negative ones (Radhakrishnan, et al., 2011). Although many studies have focused on its antibacterial effects, minimal studies have been done on its antiviral properties. A previous study has shown some antiviral activity against different strains of influenza virus (Parvez et al., 2019). In that study, several compounds were studied and Embelin was the most prominent antiviral against the influenza virus. It showed a selectivity index (SI) of 32 in the influenza virus A/Puerto Rico/8/34 (H1N1) strain. SI is the ratio of

availability concentration over antiviral effects (Flamand et al., 2014). Also, it showed an SI of 31 against A/mallard/Pennsylvania/10218/84 (H5N2) and 5 against A/Aichi/2/68 (H3N2) virus. In another study examining the hepatitis B virus, Embelin showed over 60% inhibition (Parvez et al., 2019).

Due to its bioactivity and safe consumption, Embelin is an ideal antiviral compound to examine its potential as a novel therapeutic treatment to inhibit HSV-1. The goal of this study is to investigate the antiviral effects of Embelin on HSV-1 in cultured Vero cells.

Materials and Methods

Cell Culture

Vero cells, derived from the kidney of an African green monkey, were kept in Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 5% fetal bovine serum (Serum Source International) and 1% gentamicin sulfate (10 mg/mL, BioWhittaker), and incubated at 37 °C and 5% CO₂. Cell culture was done in T25 flasks (25cm²), 6-well plates (9.5cm²) and 96-well plates (0.32cm²). The cells were incubated until 70-80% confluency was reached. This was followed by infection and treatment. A Biostar inverted microscope (Reichert) was used through all experiments requiring light microscopy.

Preparation of virus

A recombinant strain of HSV-1, GHSV-UL46 (ATCC VR-1544), was used in all the experiments performed. This strain is labeled with green fluorescent protein through

fusion of GFP to the structural tegument protein VP11/12 (Willard, M., 2002). Virus stocks were prepared by infection of a flask of Vero cells for one hour at 37 °C and 5% CO₂, followed by aspiration of the unabsorbed virus with the media. When cytopathic effect was seen, the contents of the flask were harvested and centrifuged at low speed for 5 minutes. The supernatant containing purified virus was aliquoted into 1.5 mL cryogenic tubes and stored at -80 °C.

Embelin

Purified Embelin (generously donated by Dr. M. Rossi, Vassar College, Poughkeepsie, NY) was obtained as an orange compound and dissolved in DMSO to prepare 10mM stock. Concentrations ranging from 10 µM to 70 µM were made as necessary using DMEM and were stored at 4 °C.

Cytotoxicity assays

To examine the cytotoxicity of Embelin on the cells, Vero cells were cultivated for 24 hours in a 6-well plate until 70-80% confluent. Then, increasing concentrations of Embelin were added ranging from 10µM to 70µM. After 1 hour of incubation, the solution was aspirated, and fresh media was added. Fresh media was also added to empty wells as a blank. Then the cells were observed using an inverted microscope. For quantitative analyses, a CellTiter 96 AQueous Cell Viability Assay (Promega) was utilized in a 96-well plate. This assay measures cell viability by the reduction of MTS tetrazolium to formazan, where absorbance of 490 nm is related to cell count. To perform this assay, 20µL of the MTS/PMS solution were added to the existing media in each well as per manufacturer's directions, and the plate was incubated at 37 °C and 5% CO₂ for

one hour. The absorbance was measured using 2000 PRO microplate reader (Tecan). Cell viability for each treatment condition was then calculated using the following formula: % Viability = $[(\text{Treated cells} - \text{Blank})/(\text{Cells only} - \text{Blank})]*100\%$. Standard deviation was calculated using excel function

Antiviral assay

To determine Embelin's antiviral effects on HSV-1, Vero cells were cultivated for 24 hours in a 6-well plate until 70-80% confluent. Embelin treated virus was added to the cells. The virus was treated with increasing concentrations of Embelin up to 54 μ M. Then, one-hour post infection, any unabsorbed virus media was aspirated and replaced with DMEM. Cells were incubated at 37°C and 5% CO₂. After 48 hours, the cell viability was measured using the CellTiter 96 AQueous Cell Viability Assay (Promega) as described above. In addition, a viral ToxGlo ATP Detection Buffer (Promega) assay was done. 100 μ L was added to the cells and the plate was allowed to incubate for 15 minutes as per manufacturer's directions. A 2000 PRO microplate reader (Tecan) was used. Cell viability was then plotted against HSV-1 treatment using the following equation: % Viability = $[(\text{Cells infected with treated HSV-1} - \text{Blank})/(\text{Cells only} - \text{Blank})]*100\%$. Standard deviation was calculated using excel function.

Binding assay

Vero cells were grown for 24 hours in a 96-well plate, until 70-80% confluent. The plate was pre-incubated at 4 °C for 30 minutes. Subsequently, the media was removed from the cells. Treated and untreated HSV-1 virions were incubated at room temperature of one hour followed by infection of the cells on ice. The virus was treated

with increasing concentrations of Embelin to 54 μ M. The plate was incubated for 1 hour at 4 °C, then unbound HSV-1 was carefully aspirated from the cells using a multichannel pipette. Fresh media was added to each well. The plate was incubated at 37 °C and 5% CO₂ for 48 hours and assayed using the Viral ToxGlo assay.

Penetration and post penetration assay

Vero cells were cultivated in a 96-well plate, until 70-80% confluent. Then the media was removed from the cells and infected with 100 μ L of HSV-1. The plate was incubated at 4 °C for 2 hours, to ensure attachment but not penetration of the HSV-1. Then, at room temperature, various concentrations of Embelin were added. The plate was incubated at room temperature for 10 minutes for penetration assay and 30 minutes for post penetration assay (Harden et al., 2009). This was followed by the addition of 100 μ L of 1X phosphate buffer saline (PBS) (pH 3.0) to each well to inactivate the virions. Then, the media was removed carefully using a multichannel pipette followed by the addition of 100 μ L of fresh 5% FBS DMEM. Finally, after 48 hours, viability was determined using the Viral ToxGlo ATP Detection Buffer (Promega) assay. The plate was read using the Infinite 2000 PRO microplate reader (Tecan).

Fluorescent microscopy

To prepare cells for microscopy, the cells were grown on glass cover slips in 6-well plates. Vero cells were incubated at 37 °C and 5% CO₂ until 70-80% confluent. When confluent, the cells were infected with treated HSV-1. The virus was treated with 54 μ M of Embelin for one hour. Uninfected cells served as a negative control. Untreated

virus served as positive control. After one hour of infection the media was aspirated, and new media was added. Then the cells on the slip were prepared and fixed 8 hours post infection (hpi), and 12 hpi. First, the cells were treated with 300 μ L of DAPI (4,6 - diamidino - 2 - phenylindole) stain for 5 minutes at 37°C in the dark. Then, the cells were fixed using 2-4% paraformaldehyde for 10-20 minutes followed by PBS wash. Then mounting medium was used to fix coverslip on the slide. The mounting medium solution contained 90% glycerol and 10% PBS. The slides were observed using Zeiss Axio Scope A1 microscope in the dark. The images were merged using imageJ software.

Quantitative polymerase chain reaction (qPCR)

To prepare for qPCR, the cells were grown in a 6-well plate at 37 °C and 5% CO₂ until 70-80% confluent. Then, the cells were infected with treated HSV-1. The virus was treated with 54 μ M of Embelin for an hour. Uninfected cells served as a negative control. Untreated virus served as positive control. After 30 minutes of infection the media was aspirated, and new media was added. 12hpi the DNA was collected using Dneasy blood & tissue kit (QIAGEN). PCR followed by gel electrophoresis was performed to confirm the presence of viral DNA. The primers HSV1 gD forward 5'-CAACCCTACAACCTGACCATC-3' and HSV1 gD reverse 5'-TTGTAGGAGCATTCGGTGTAC-3' were used. Then qPCR was performed using Applied Biosystems™ SYBR™ Green PCR Master Mix on 96-well plate MicroAmp™ Optical 96-Well Reaction Plate. Also, a 10-fold serial dilution was done up to the 10⁻⁵ dilution in order to determine the standard curve equation and R². This calculation was performed using the excel function.

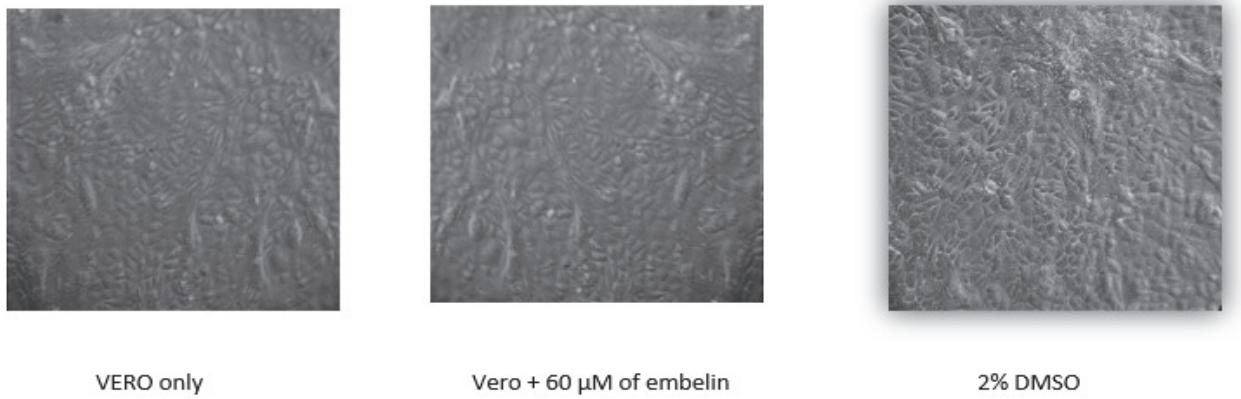
Results

Cytotoxicity Study of Treatment of Vero cells with Embelin

The cytopathic effects of Embelin and DMSO were determined by treatment of these cells with increasing concentrations up to 60uM of Embelin and 2% of DMSO. The qualitative assessment was established, by determining the morphology of the cells through inverted microscope imaging. Cells treated with up to 60μM showed no signs of morphological change and the cell monolayer was intact. This was compared to the cells only control (Figure 1 a). The effects were quantified through the MTS assay. The cells were grown in a 96-well plate and incubated for 1 hour with increasing concentrations of Embelin. The assay was performed after 56 hours of incubation and absorbance was measured at 490 nm which directly correlates with cell viability. Percent viability ranged from a low of 84.21% at 50 μM to 100% for 54 μM concentrations (Figure 1b). This difference might be due to the nature of the assay reading with a small number OD. Calculation was performed using $\% \text{ Viability} = [(\text{Cells infected with treated HSV-1} - \text{Blank}) / (\text{Cells only} - \text{Blank})] * 100\%$. The bars demonstrate the standard deviation was calculated using excel function. This demonstrates the Embelin does not exert toxic effects up to 70uM concentrations.

Figure 1:

(a)



b)

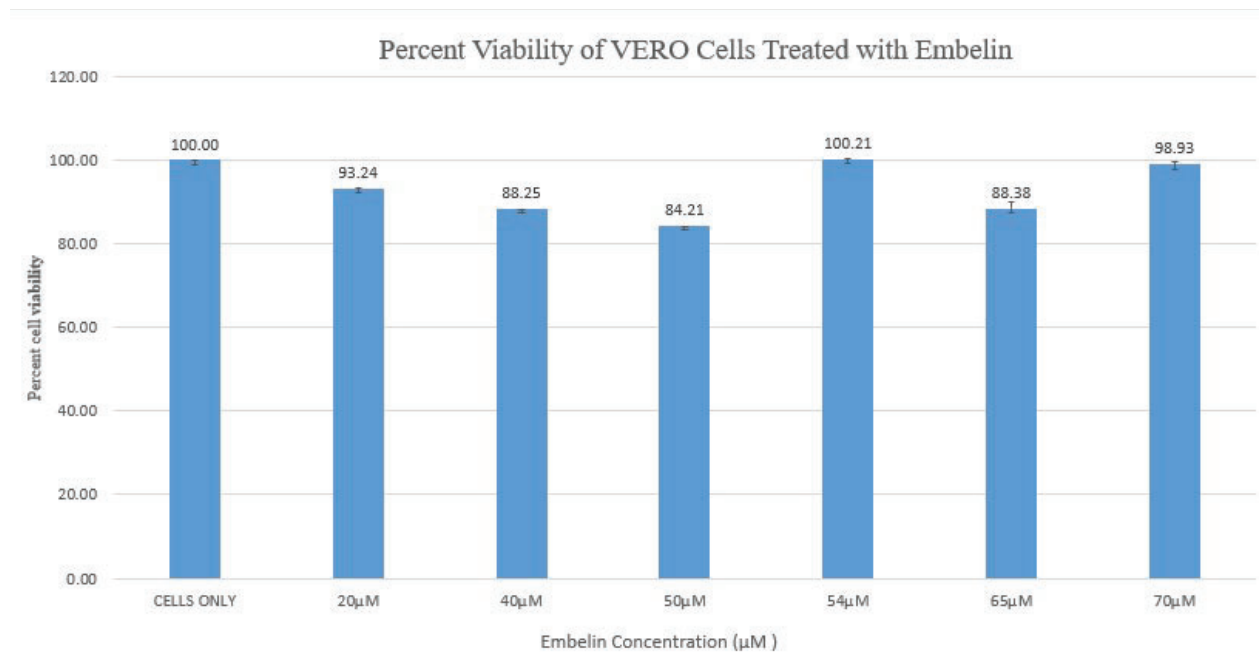


Figure 1: (a) presents the viability of Vero cells with Embelin treatments at 60 μM . Images were taken through a Biostar inverted microscope (Reichert) following 48 hrs incubation at 37 °C and 5% CO₂. Left images show cells only where the morphology of the cells shows a healthy, intact monolayer. Middle image shows no change in morphology of the Vero cells even after 60 μM of Embelin treatment after

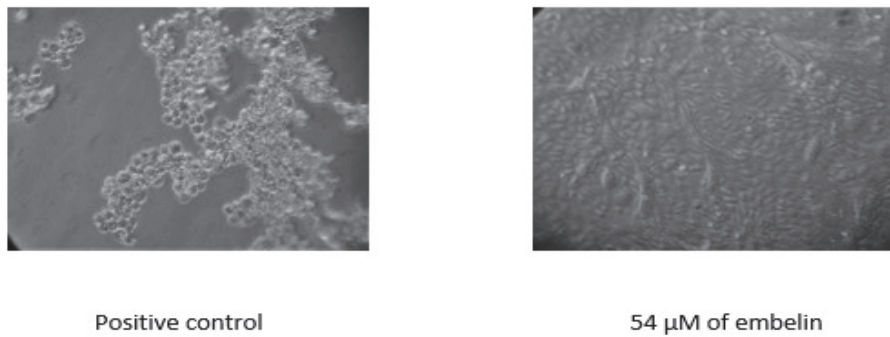
48hours. Right image shows the administration of 2% DMSO presenting no toxicity for the vehicle solution. (b) Displays cell viability after increasing concentration of Embelin treatment after 56hours. The error bars demonstrate the standard deviation calculated through excel function. Cell viability was calculated in relation to absorbance through % Viability = $[(\text{Cells infected with treated HSV-1 absorbance} - \text{Blank absorbance}) / (\text{Cells only absorbance} - \text{Blank absorbance})] * 100\%$; where Blank absorbance is the media only samples.

Antiviral effects of Embelin on HSV-1

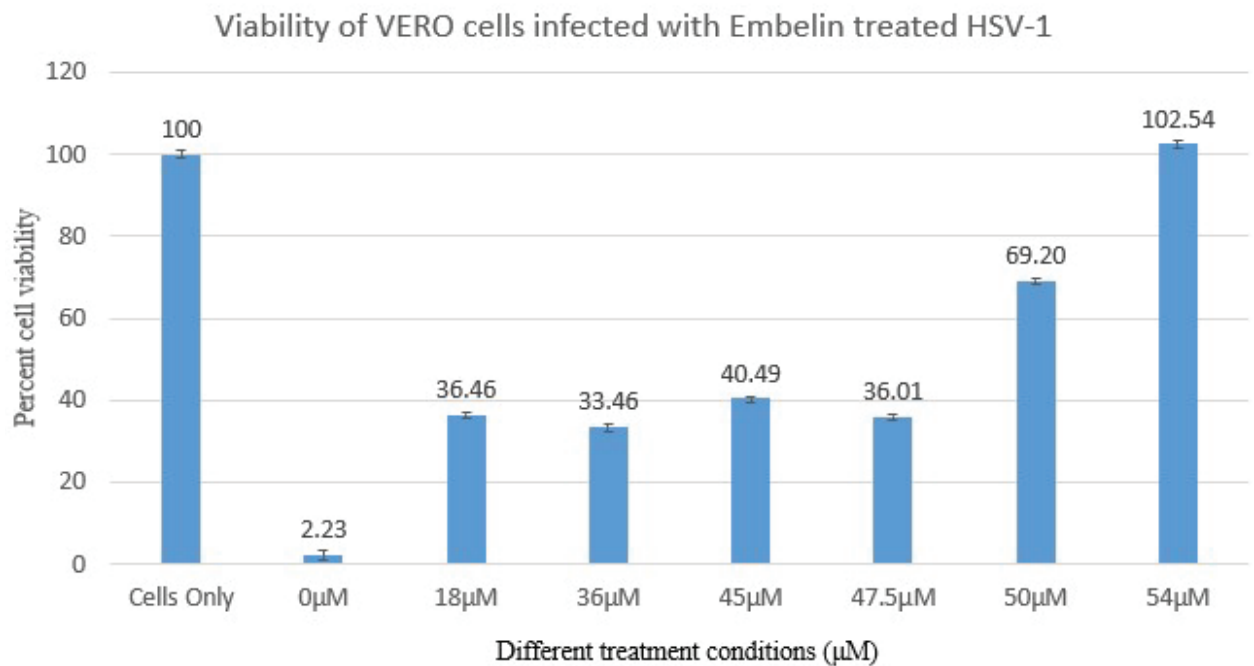
To demonstrate the antiviral effects of Embelin on HSV-1, the virus was treated with Embelin for 1 hour then the virus was allowed to infect the cells. Increasing concentrations of Embelin was administered where 54 μ M was the most effective (Figure 2a right). Untreated HSV-1 (positive control) results in rounding and lifting of the Vero cell monolayer (Figure 2a left). The data demonstrated dose-dependent antiviral effects with the highest inhibition at 54 μ M (Figure 2b). This concentration had 102.5% cell viability. This means that it had slightly higher absorbance than the cell only sample. Then, percent inhibition was calculated from the formula = $100 \times [1 - (\text{Embelin treatment} - \text{cells only}) / (\text{cells only} - \text{no treatment})]$. The percent inhibition reached over 100% inhibition with the 54 μ M sample and 68% with the 50 μ M treatment (Figure 2c). Then to confirm this observation, the procedure was performed again using Viral ToxGlo ATP Detection Buffer (Promega). The results, consistent with the previous observations, showed 54 μ M to have the highest cell viability (Figure 2d). This was done through measuring the luminescence 48hpi. No significant difference was seen at the

54 μ M treatment unlike the rest. Significance was calculated using the student T test function in Excel. Percent inhibition was calculated for each concentration of Embelin (Figure 2f). The highest inhibition was observed through the 54 μ M treatment at over 98% inhibition followed by 74% at the 50 μ M concentration. This also suggested the presence of dose-dependent inhibition.

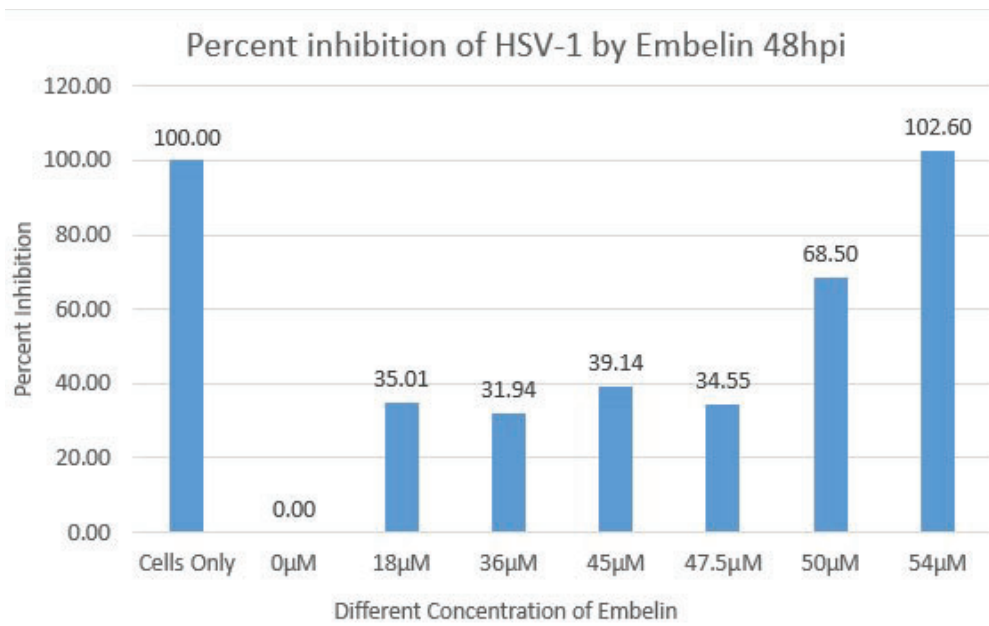
Figure 2 (a)



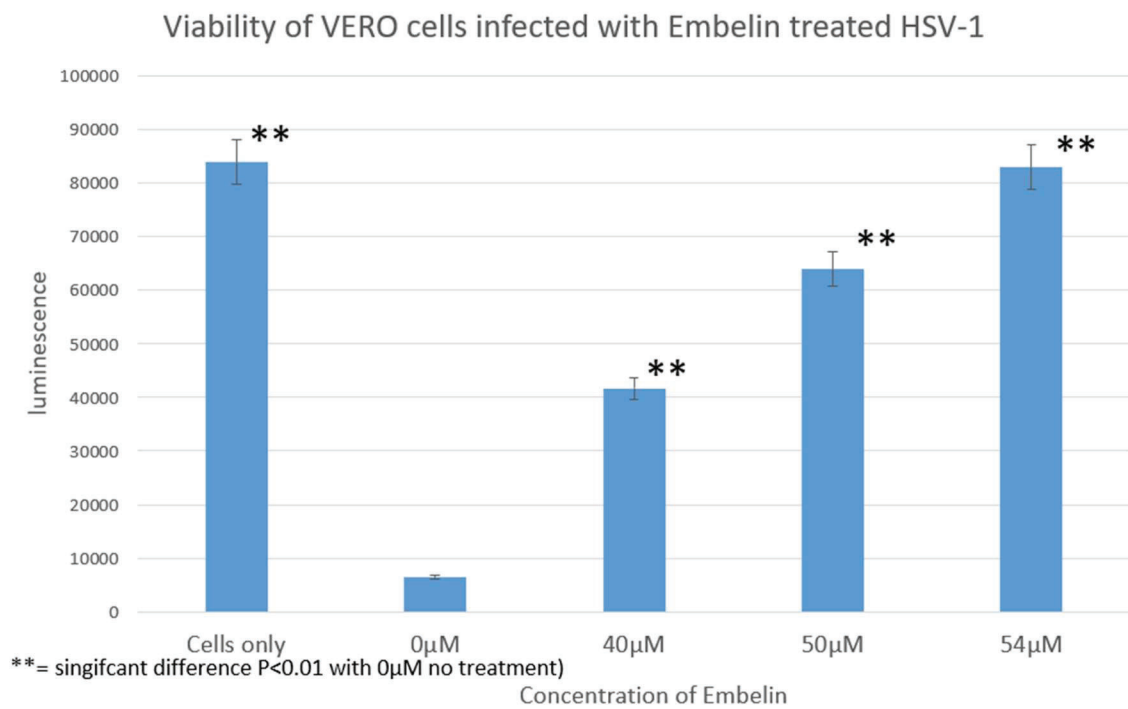
(b)



(c)



(d)



(e)

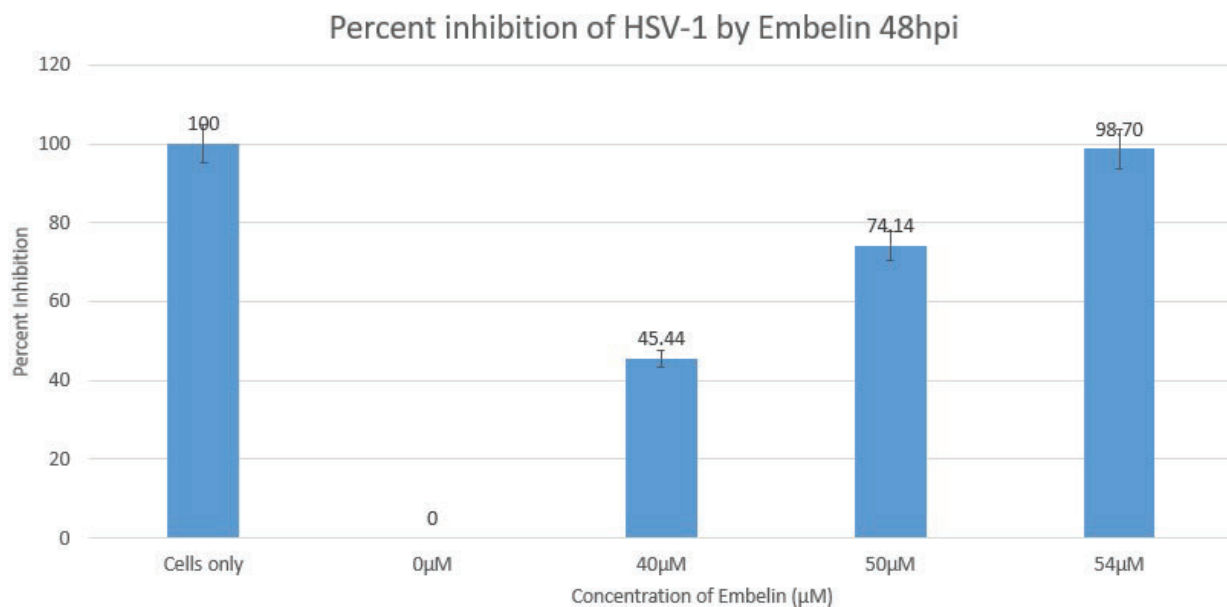


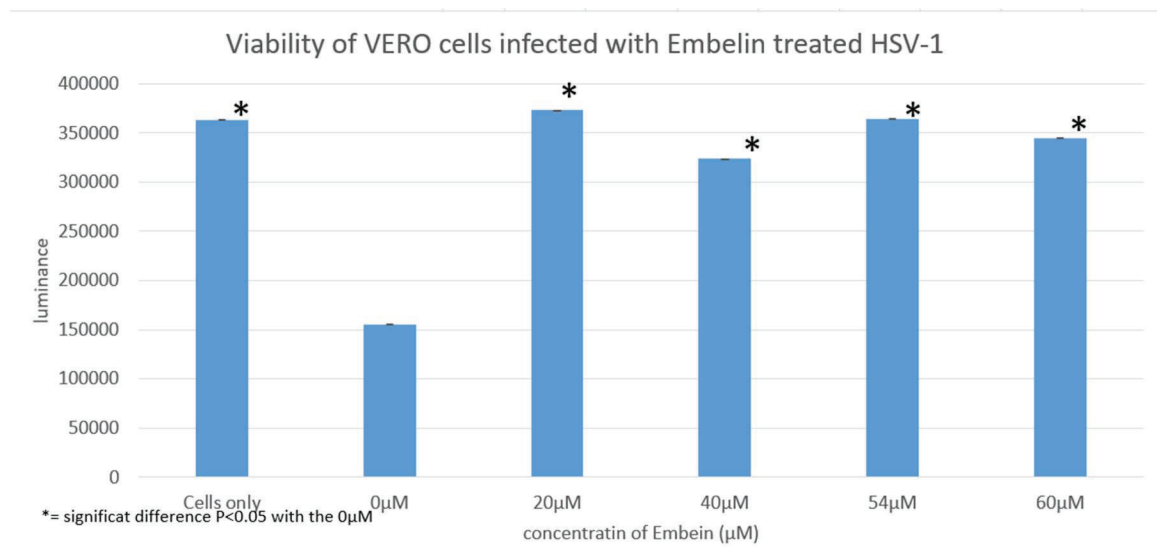
Figure 2: (a) Presents the antiviral activity of Embelin after 48 hpi. The cytopathic effect (left) can be seen after HSV-1 infection of Vero cells. 54μM treatment of HSV-1 (right) indicated no appearance of cytopathic effect. (b) Cell viability after CellTiter 96 AQueous Cell Viability Assay (Promega) was utilized 48hpi. This demonstrated dose-dependent inhibition with cell viability increasing with increasing concentration of Embelin. (c) The percent of antiviral inhibition of Embelin concentrations on HSV-1, 54μM treatment had the highest with 100% inhibition. (d) Viral ToxGlo ATP Detection Buffer (Promega) showed the luminescence of different viral Embelin treatment at 48hpi. (e) The percent inhibition after Embelin treatment. Consistent with previous data, 54μM had the highest inhibition over 98% followed by 50μM at around 74% inhibition.

Inhibition of binding by Embelin

To investigate the antiviral activity of Embelin on the binding step of the viral cycle, HSV-1 was treated at increasing concentrations of Embelin and then used to infect the Vero cells for 1 hour at 4 °C. This will allow for the binding to occur but not the penetration of the virus. After 48 hpi, luminescence detection was done through the Viral ToxGlo Assay (Promega). This demonstrated, unlike the previous studies, no clear pattern in inhibition (Figure 3 a). Yet, there were clear signs of inhibition when antiviral activity was calculated with the lowest having 80% inhibition (Figure 3b). This indicates that Embelin inhibits the binding stage of HSV-1 infection cycle.

Figure 3

(a)



(b)

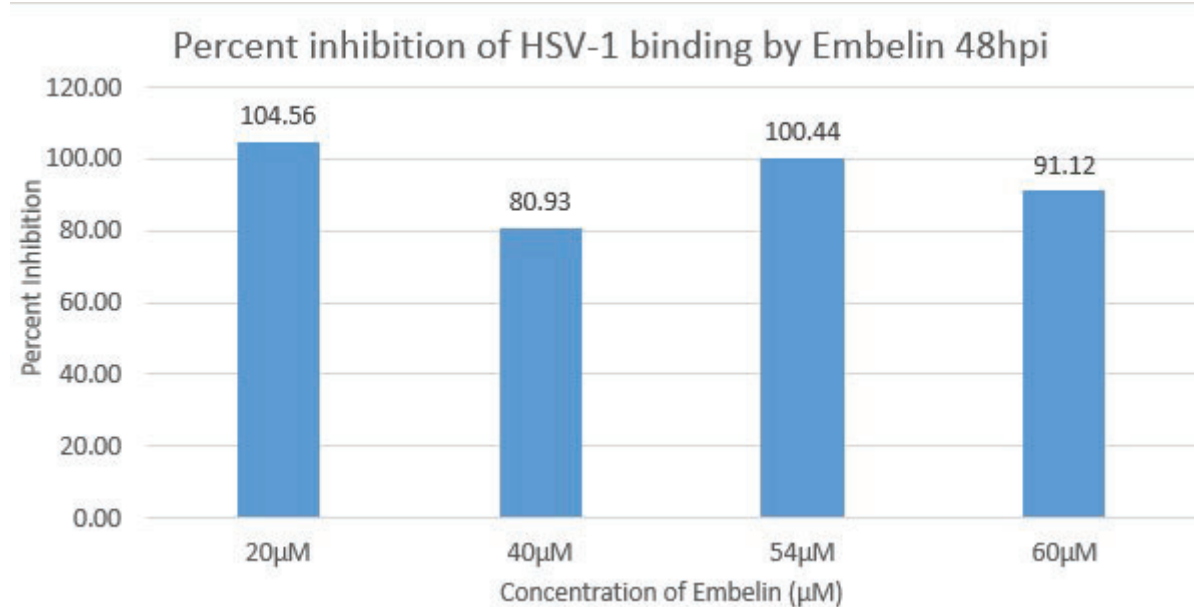


Figure 3: The antiviral effects of Embelin on the binding step of the viral cycle. (a)

Luminescence measurements of Embelin treated virus infection. The error bars represent the standard deviation which were determined through the STDEV.S function in Excel

(b) Demonstrates the percent inhibition of the virus by Embelin at different concentrations. The percent inhibition ranged from 80.93 at 40 µM concentration of Embelin to 104.56 percent inhibition at 20µM concentration of Embelin.

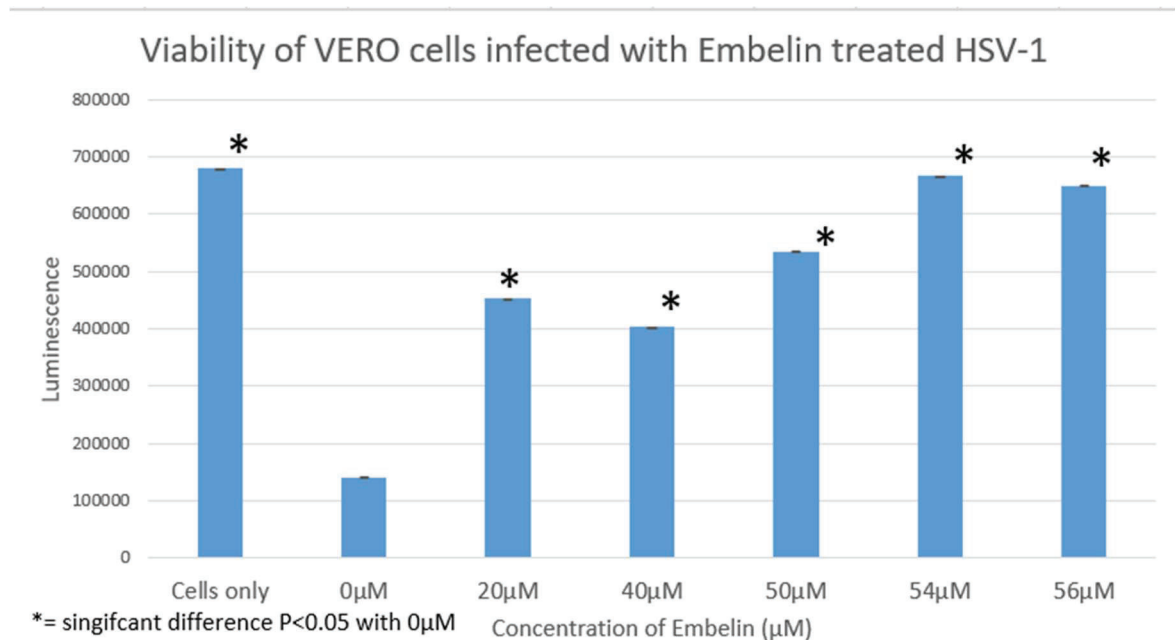
Inhibition of penetration by Embelin

In order to determine the antiviral effects of Embelin at the time point of penetration of the virus into the host cell, a 96-well plate of Vero cells were infected with HSV-1 at 4 °C for 2 hours, to ensure attachment but not penetration of the HSV-1. Then a treatment of 10 minutes was performed at varying concentrations of Embelin at room temperature, during the penetration step. After the addition of 100 µL Viral ToxGlo ATP Detection Buffer (Promega), Luminescence was measured. Dose dependent effects were

observed (Figure 4a). Luminescence, which is correlated with cell viability, increased as Embelin concentration increased. Highest cell viability occurred when HSV-1 was treated at 54 μ M and 56 μ M concentrations, with no significant difference determined between these and the control. When the antiviral activity was calculated, 54 μ M and 56 μ M treatments had the highest percent inhibition reaching over 97% and 94% respectively (Figure 4b). This demonstrates that Embelin has antiviral activity in the penetration step of the viral cycle. The data indicated that treatment of HSV-1 with Embelin antiviral activity during the binding stage (Figure 3a and b) as compared to the penetration stage (Figure 4a and b).

Figure 4:

(a)



(b)

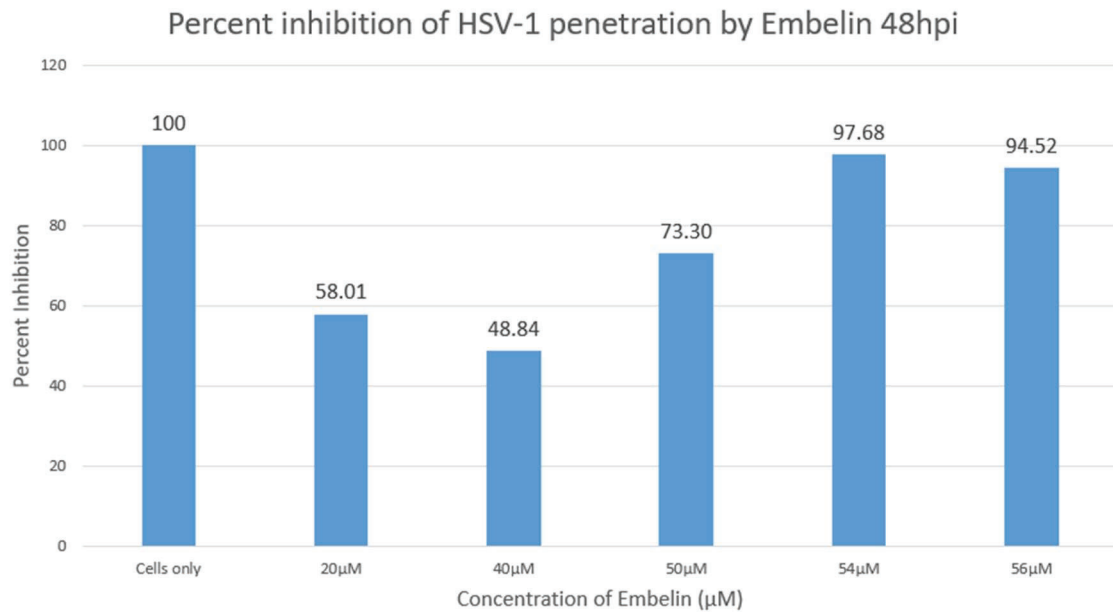


Figure 4: The antiviral effects of Embelin on the penetration step of HSV-1. (a) Displays the results of the luminescence measurement determined by the Viral ToxGlo ATP Detection Buffer assay (Promega) 48hpi. Standard deviation of four wells were calculated and corrected by subtracting the blank signal; where the error bars represent that deviation calculated through STDEV.S function in Excel. (b) The antiviral percent inhibition of Embelin.

Inhibition of post penetration by Embelin

The antiviral effects of Embelin on the post penetration step of the viral cycle was determined through infection of Vero cells in a 96-well plate. After confluency was observed, the cells were infected with HSV-1 at 4 °C for 2 hours, to ensure attachment but not penetration of the HSV-1, then treated after 30 minutes of incubation 37 °C to ensure that the virus had entered the cell. Then, 48hpi, the luminescence was measured

following the addition of 100 μ L Viral ToxGlo ATP Detection Buffer (Promega). The results indicated that treatment with Embelin did not affect viability after the virus was allowed to enter the cells (Figure 5).

Figure 5:

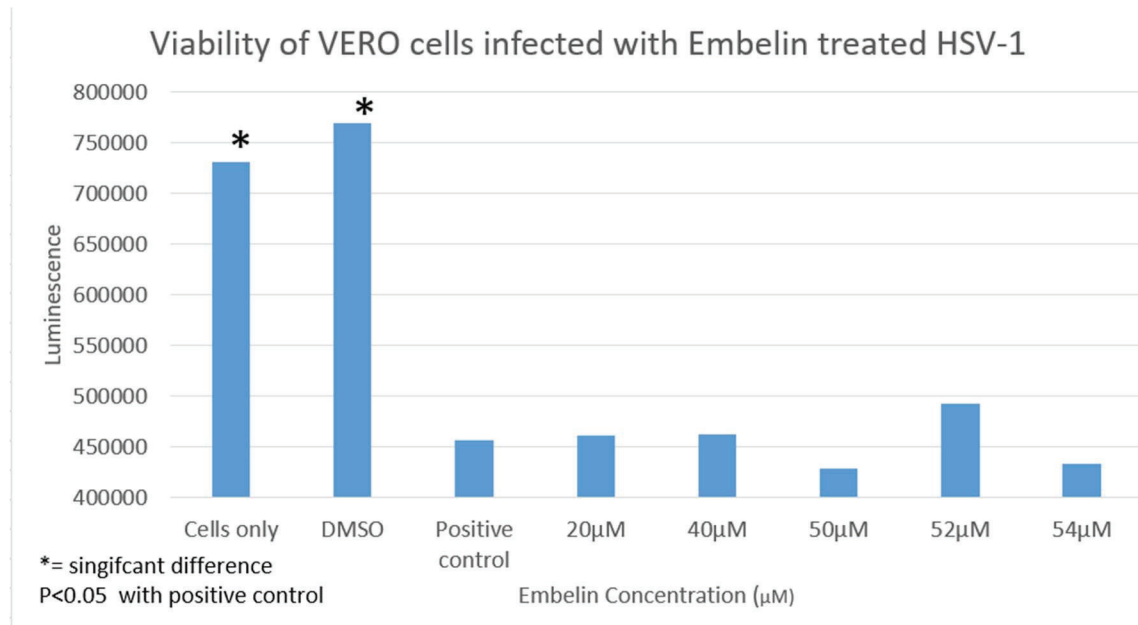


Figure 5: Presents the antiviral effects of treatment of HSV-1 with concentrations of Embelin ranging from 20 μ M to 54 μ M post-penetration. Positive control represents no treatment. DMSO represents the vehicle control up on 2% administration of DMSO.

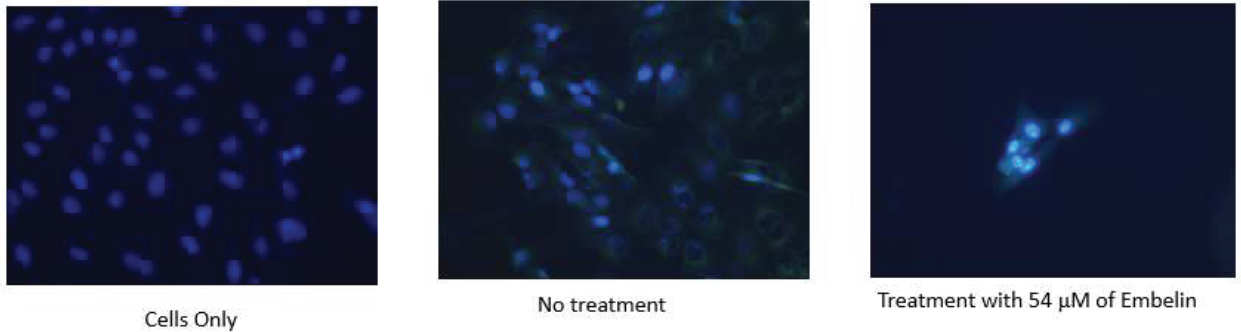
Fluorescence microscopy

The HSV-1 strain contains GFP fusion to the structural tegument protein VP11/12. This enabled visualization of the effects of treatment of HSV-1 with Embelin through fluorescent microscopy 8hpi and then 12hpi. Infected cells were visualized with fluorescent microscope (Figure 6). Separate images were taken of GFP and DAPI and merged using imageJ. GFP (green) represents the virus and DAPI (blue) represents the nucleus of the host cells. It is noticeable that there is a minor decrease in the amount of

GFP in the no treatment image compared with the Embelin-treated HSV-1 at 8hpi (Figure 6a). However, there is a greater difference in the expression of GFP between the untreated and treated HSV-1 at 12hpi (Figure 6b).

Figure 6:

(a) Fluorescence microscopy of Embelin-treated HSV-1, 8 hours post infection



(b) Fluorescence microscopy of Embelin-treated HSV-1, 12 hours post infection

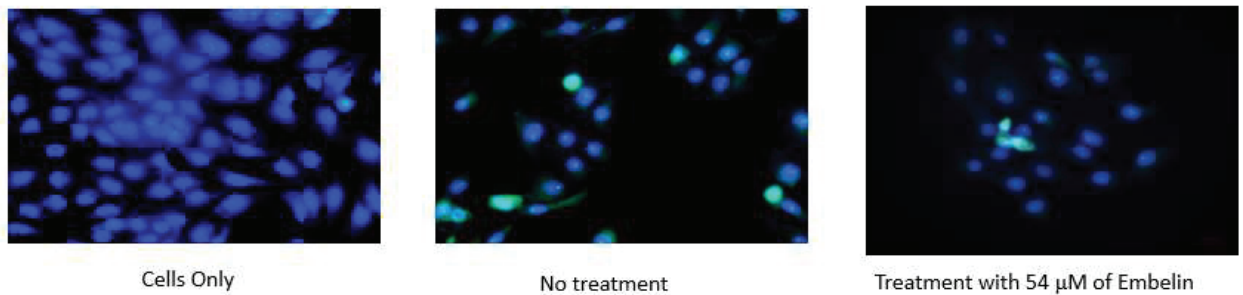
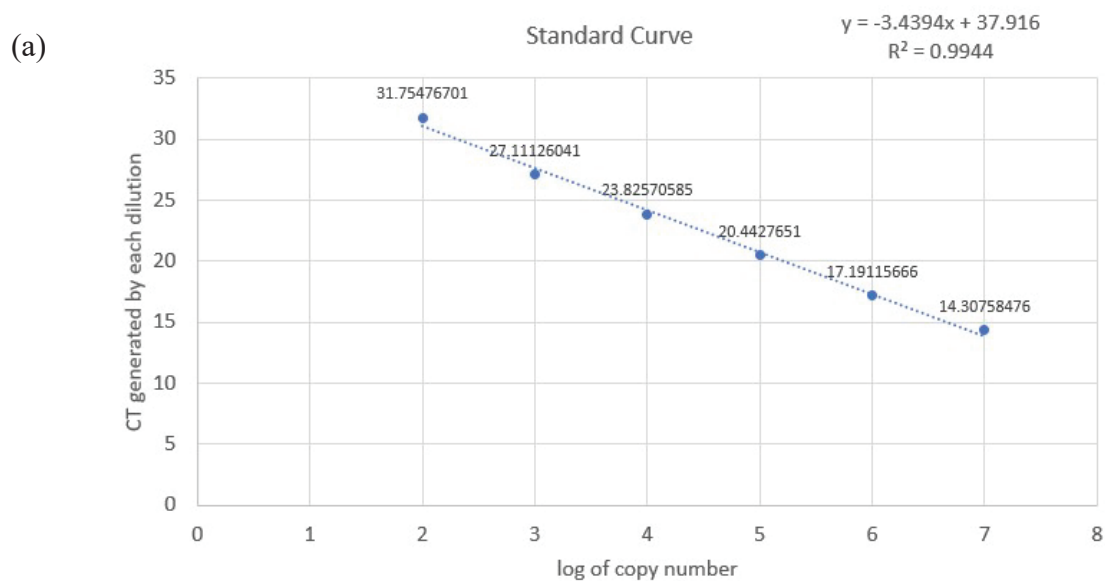


Figure 6: Fluorescent microscopy images of Vero cells only (left), HSV-1 infected cells (middle) and HSV-1 treated with 54 μ M Embelin (right). Viral particles with GFP appear green and the Vero cell nuclei are blue. (a) Images taken of the cells 8hpi and (b) images taken of the cells 12hpi.

Quantitative Polymerase Chain Reaction (qPCR)

In order to further investigate the antiviral effects of Embelin on HSV-1, qPCR Vero cells were cultivated in 6-well plates followed by infection once confluent. The DNA was extracted and analyzed by PCR to confirm the presence of the viral DNA (data not shown) and qPCR. The number of and viral particles were measured through calculation from the standard curve after 10-fold serial dilution and mathematical analysis (Figure 7a). These calculations lead to the number of viral particles present in treatment condition in contrast to no treatment condition (Figure 7b). The treatment of HSV-1 by Embelin resulted in a decrease in the viral particles 12hpi. It is important to note that these numbers represent the estimated number of viral particles that managed to enter the cells. The media was removed after the infection and only the DNA inside the cells were collected. To further investigate, the percent inhibition was calculated to compare Vero cells only and untreated HSV-1. The inhibition reached 96% just as was observed using the biochemical assays.

Figure 7:



(b)

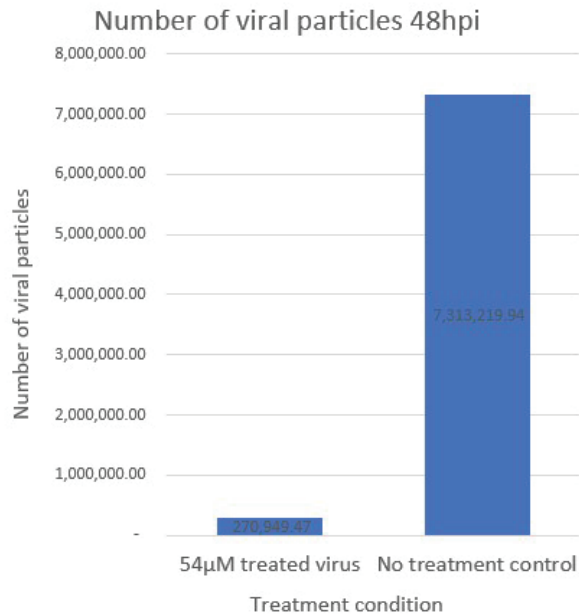


Figure 7: (a) Standard curve of the qPCR calculated from the CT value of the different dilutions. $Y=mx+b$ equation of the line was calculated using Excel so is the R^2 value. (b) Demonstrate the number of particles in the no treatment control compared to non-treated control.

Discussion and Conclusions

HSV-1 is a double stranded enveloped DNA virus that causes oral and genital herpes (Whitley et al., 2001). Currently there is no cure for HSV-1 although nucleoside analog antivirals such as acyclovir are available that shorten the span of symptoms (James et al., 2014; Tyring et al., 2000). Additionally, there is no vaccine available, and establishing one has been highly difficult (Kuo et al., 2014). Therefore, discovering a novel drug that could present some antiviral properties against HSV-1 is of necessity. Natural products have shown many antiviral properties. Embelin is a novel medicinal compound that has shown some incredible biological activity (Kumar et al., 2017). However, there are minimal studies that examined the antiviral effects against viruses with none that has examined effects on HSV-1. Therefore, this study focused on the investigation of the effects of Embelin treatment on HSV-1 infection and then further

investigated the inhibition at various stages of the viral cycle. Finally, the data was further supported using both fluorescent microscopy and qPCR.

In this study, it was determined that Embelin has no toxic effects on the cell up to 70 μ M (Figure 1b). This finding aligned with previous studies that presented Embelin as a safe compound that can be used up to 200mg/Kg (Mahendran et al., 2011). Therefore, there was no need to examine the maximum amount of Embelin that could be used since it was determined from this study and previous studies that a high concentration of this compound can have no toxic effects. Also, the goal of identifying an antiviral therapeutic is to find the lowest inhibitory concentration. Qualitative antiviral effects were observed using microscopy at concentrations as low as 20 μ M, followed by further quantitative support from both the MTS and Tox-Glo assays. Inverted microscopy revealed the cytopathic effects of the virus on the cells and the effect of viral treatment with Embelin (Figure 2a). When the MTS assay was performed, the treated sample demonstrated 102% cell viability (i.e. had more signal than the cells only control) (Figure 2b). When the percent inhibition was calculated, in both the MTS and Tox-Glo assays, the percent inhibition was the highest at 54 μ M calculated at 102% and 98.7%, respectively (Figure 2c and e). The results demonstrated that Embelin indeed has antiviral activity and it inhibited the viral infection in a dose dependent manner. However, a large difference in the inhibitory activity was observed with the small difference in concentration of 4 μ M. When comparing inhibition caused by treatment with 50 μ M and 54 μ M the percent increased by 25 - 30% in both the MTS and the ToxGlo assays consistently. It is not clear why this might occur. A possible reason might be due to its ability to disrupt the membrane (Hu et al., 2011). This was observed in a previous study on antimicrobial

cationic peptides, which usually affects the membrane, on HSV-1 (Albiol Matanic and Castilla, 2004). In the study, a similar pattern to Embelin was observed. The percent inhibition increased at specific concentrations. However, studies need to be done to further investigate this hypothesis of the action of Embelin on the membrane of HSV-1.

To further understand how Embelin might inhibit the virus, the compound was administered at various points of the viral infection cycle. Embelin had the highest percent of inhibition when administered at the binding stage of the viral cycle (Figure 3b). Although there was no dose dependent pattern or inhibition pattern, it is a promising finding since the lowest percent inhibition had over 80% at 40 μ M treatment. The penetration assay demonstrated a dose dependent pattern, (Figure 4b) yet had less inhibition compared to the binding step administration. However, the inhibition was still promising, reaching over 97% in the 54 μ M treatment. Also, there was an over 35% increase in inhibition between the 50 μ M and the 54 μ M treatment. This was consistent with the data from previous assays. When the virus was allowed to enter the cell, there was minimal to no difference between treatment and no treatment (Figure 5). This was consistent with previous antiviral assays that allowed the virus to enter the cell and then was visualized under the microscope (images not shown). There was no difference when visualized, where the cytopathic effects were still present even after treatment. These data demonstrate that Embelin is most effective at binding and penetration stages. However, when the virus is allowed to enter the cell, there is no observed inhibition.

To further support these data, the effects were visualized with fluorescence microscopy. Images were taken after the slides were fixed at 8hpi and then 12hpi. In the 8hpi treatment the virus expression (green) is reduced after Embelin treatment (Figure

6a). In the 12hpi, viral expression can also be seen to decrease (Figure 6b). When comparing (a) and (b) in Figure 6, there appears to be more expression in 12hpi as expected in both treated and non-treated assays due to the amount of time the virus was allowed to replicate in Vero cells. However, the reduced amount of virus in the treated sample as compared to non-treated is apparent.

Finally, a qPCR was performed to further demonstrate the antiviral activity of Embelin on the virus. DNA extraction was performed at 48hpi. The viral particles were calculated by determining the number of stock viral particles followed by 10-fold serial dilution of the stock up to the 10^{-5} . Then the number of viral particles in treatment and control were determined from the equation $y=mx+b$ (Figure 7a). This indicated over 7 million particles to be found in the no treatment condition inside the cell and around less than 300,000 when HSV-1 was treated with Embelin (Figure 7b). This showed 96% percent inhibition when compared with the negative and positive control. Embelin could be blocking the virus from entering the cell (as demonstrated in the binding and penetration assays) reducing its propagation to a large level. Therefore, the reason for the lower DNA count in treatment is not because Embelin could have blocked DNA synthesis of the virus, but rather Embelin inhibited the entry of the virus. Yet, this needs to be further supported by other studies and experiments.

Overall, this study shows that Embelin inhibits HSV-1 in cultured Vero cells. Embelin is most effective in the binding step of the viral cycle followed by penetration. Embelin had the greatest inhibition in concentrations above 50 μ M concentration. Embelin did not seem to have any effects against the virus once it entered the cell. This indicates a possible mechanism by which Embelin could inhibit the virus.

Although this study demonstrated these findings through multiple means, further studies are suggested, including a possible study of how Embelin might affect the membrane of the virus. Also, investigate the effect of longer treatment times for the cells at lower concentrations of Embelin. Finally, the antiviral effects of Embelin on naked membrane-less viruses should be carried out as well as *in vivo* studies.

Overall, this study demonstrated that Embelin has antiviral effects against HSV-1 in cultured Vero cells. This could lead to an effective therapeutic treatment to reduce the spread of HSV-1.

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